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1. Waldegger et al., PNAS USA, 94, 4440-4445, 1997.
2. Webster et al., Mol. Cell. Biol., 13, 2031-2040, 1993.
3. Adams et al., Nature 377 (6547 Suppl), 3-174, 1995.

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## Characterization of *sgk*, a Novel Member of the Serine/Threonine Protein Kinase Gene Family Which Is Transcriptionally Induced by Glucocorticoids and Serum

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A novel member of the serine/threonine protein kinase gene family, designated *sgk*, for serum and glucocorticoid-regulated kinase, was identified in a differential screen for glucocorticoid-inducible transcripts expressed in the Con8.hd6 rat mammary tumor cell line. *sgk* encodes a protein of 49 kDa which has significant sequence homology (45 to 55% identity) throughout its catalytic domain with *rac* protein kinase, the protein kinase C family, ribosomal protein S6 kinase, and cyclic AMP-dependent protein kinase. *sgk* mRNA is expressed in most adult rat tissues, with the highest levels in the thymus, ovary, and lung, as well as in several rodent and human cell lines. *sgk* mRNA was stimulated by glucocorticoids and by serum within 30 min, and both inductions were independent of *de novo* protein synthesis. The transcriptional regulation by glucocorticoids is a primary response, since the promoter of *sgk* contains a glucocorticoid response element consensus sequence 1.0 kb upstream of the start of transcription which is able to stimulate chloramphenicol acetyltransferase reporter gene activity in a dexamethasone-dependent manner. Antibodies that specifically recognize *sgk*-encoded protein on an immunoblot were generated. This protein was shown to increase in abundance with glucocorticoid treatment in a manner which paralleled the mRNA accumulation. This is the first report of a presumed serine/threonine protein kinase that is highly regulated at the transcriptional level by glucocorticoid hormones and suggests a novel interplay between glucocorticoid receptor signaling and a protein kinase of the second messenger family.

Glucocorticoid hormones play a fundamental role in the control of homeostasis, differentiation, and development in animal tissues (8, 10, 15, 29, 35). These steroids exert their regulatory effects through intracellular receptors which act as potent transcriptional activators of genes that possess glucocorticoid response elements (GREs) (reviewed in references 3 and 11). They can also negatively regulate the expression of a number of genes, by interfering with the actions of the AP-1 transcriptional complex (9, 17, 31, 40). Beyond these primary responses, glucocorticoids may exert some of their pleiotropic actions through the induction or repression of regulatory genes, whose products could in turn influence the expression or activity of a wide variety of cellular components. Relatively little is known, however, about signaling pathways under the direct transcriptional control of glucocorticoids.

We have previously shown that one of the actions of glucocorticoids is to strongly inhibit both the *in vitro* and *in vivo* growth of Con8.hd6 rat mammary tumor cells, which are a derivative of the DMBA-induced 13762NF adenocarcinoma (37). Cell fusion experiments supported the idea that glucocorticoids inhibit the proliferation of these cells by inducing dominant *trans*-acting regulatory factors (38). As a test of this hypothesis, differential screening of a cDNA library generated from mRNA expressed in glucocorticoid-treated Con8.hd6 cells was used to identify and characterize genes which are transcriptionally stimulated by glucocorticoids in these cells.

In this study, one of the genes shown to be transcriptionally regulated by glucocorticoids, *sgk*, encodes a novel

putative member of the serine/threonine protein kinase family. Phosphorylation-dephosphorylation networks are commonly utilized by extracellular regulators, such as growth and differentiation factors, to rapidly and reversibly transduce signals from the extracellular environment to the cytoplasm and nucleus (reviewed in references 27 and 39). This report presents the first evidence of transcriptional regulation of a protein kinase by glucocorticoids, and we speculate that *sgk* could be involved in mediating some of the cellular functions of glucocorticoid hormones. We have also found that serum rapidly stimulates *sgk* transcript levels in the absence of glucocorticoids. As a first step in examining its possible role in cell proliferation and other glucocorticoid-regulated pathways, this report describes the isolation and characterization of *sgk* and its induction by glucocorticoids and serum.

### MATERIALS AND METHODS

**Cells and materials.** Con8.hd6 mammary cells are derived from a DMBA-induced mammary adenocarcinoma and have been described previously (38). Cells were routinely cultured in Dulbecco's modified Eagle's medium (DME-F12 (50:50) containing 10% calf serum; DME-F12, actinomycin D, cycloheximide, and steroid hormones were purchased from Sigma Chemical Co. (St. Louis, Mo.). Oligonucleotides used for sequencing and primer extension were prepared at the oligonucleotide synthesis facility (University of California, Berkeley). Radionucleotides ( $\alpha$ -<sup>32</sup>P]dCTP (3,000 Ci/mmol) and [<sup>35</sup>S]methionine (1,150 Ci/mmol) were obtained from Dupont/NEN (Boston, Mass.). All other reagents were of the highest available purity.

Generation of a cDNA library representing glucocorticoid-

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induced transcripts. Cytoplasmic RNA was isolated from Con8.hd6 cells grown for 48 h in DME-F12 (50:50), 10% calf serum, and 1  $\mu$ M dexamethasone as previously described (38). mRNA was selected from the cytoplasmic RNA by two passes over an oligo(dT)-column (Collaborative Biomedical Products, Bedford, Mass.), and cDNA was synthesized as recommended by the kit manufacturer (Amersham, Arlington Heights, Ill.), using avian myeloblastosis virus reverse transcriptase and oligo(dT) primers. Double-stranded cDNA was methylated with *EcoRI* methylase and ligated to phosphatized *EcoRI* linkers (Stratagene, La Jolla, Calif.), which were then activated by *EcoRI* digestion according to standard procedures. Linkers were removed, and the cDNA was size selected by fractionation on a Bio-Gel A50M column (Bio-Rad, Richmond, Calif.). Approximately 1  $\mu$ g of double-stranded cDNA ranging in size from 0.5 to 6 kb was recovered. Three hundred nanograms of cDNA was ligated to 3- $\mu$ g of *EcoRI*-digested, phosphatized lambda ZAPII phagemid arms (Stratagene). The ligated products were packaged by using Stratagene GigapackII extract and plated onto *Escherichia coli* XL-1 Blue cells (Stratagene). This library was estimated to contain  $3 \times 10^5$  individual plaques, with about 25% nonrecombinants. The library was amplified once and stored at 4°C.

**Differential screening of cDNA library for dexamethasone-induced genes.** The lambda library was plated onto *E. coli* XL-1 Blue at a density of 3,000 to 5,000 plaques per 150-mm-diameter plate. Plaques were transferred to duplicate nylon filters (Micron Separations, Inc., Westboro, Mass.), and the DNA was denatured by placing the filters plaque side up in 0.2 M NaOH–2.5 M NaCl for 2 min. The filters were neutralized by immersion first in 0.4 M Tris (pH 8.0)–2 $\times$  SSC (1 $\times$  SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and then in 2 $\times$  SSC for 2 min each time. DNA was fixed onto the membranes by UV cross-linking (Stratagene Stratatinker). Filters were prehybridized for 2 h in a solution containing 5 $\times$  SSC, 5% Denhardt's solution, 0.1% sodium dodecyl sulfate (SDS), and 100  $\mu$ g of sheared salmon sperm DNA per ml at 65°C. mRNA was isolated as described above from Con8.hd6 cells grown in medium either containing dexamethasone for 48 h or without hormone. To generate probes, single-stranded cDNA was prepared as described for the cDNA library, and the cDNA was radiolabeled to high specific activity by using a random primer labeling kit (Amersham). The respective probes ( $10^6$  cpm/ml) were added to the duplicate filters and allowed to hybridize for 16 to 20 h at 65°C. Washing and autoradiography were as described for RNA hybridization analysis. Plaques corresponding to signals visibly stronger with the dexamethasone treatment (+Dex) probe were corelated, replated, and rescreened until individual positive phage could be obtained. pBluescript plasmids were excised from the lambda ZAPII phagemid by using R408 helper phage as recommended by the manufacturer, and the inserts were isolated by *EcoRI* restriction enzyme digestion.

**Sequence analysis and alignment.** cDNAs isolated from the differential screen were sequenced by the dideoxy method, using the Sequenase version 2.0 kit (United States Biochemicals, Cleveland, Ohio). The sequence of full-length *sgk* cDNA was obtained on both strands from a combination of overlapping subclones, restriction fragment subclones, and exonuclease III-mung bean nuclease deletions (Stratagene). GC-rich regions were resolved with ITP. The GenBank data base was searched for homologous sequences, using the FASTA computer program (26), and aligned by using the

Genalign multiple sequence alignment program from Intelli-genetics.

**Primer extension.** Primer extension was performed essentially as described previously (1). Briefly, 100 ng of an oligonucleotide complementary to nucleotides +45 to +79 of *sgk* was labeled at the 5' end with [ $\gamma$ - $^{32}$ P]ATP and T4 polynucleotide kinase, and the oligonucleotide was precipitated three times with 2.5 M ammonium acetate and ethanol to remove unincorporated nucleotides. Then  $5 \times 10^6$  cpm of labeled oligonucleotide was mixed with 10  $\mu$ g of poly(A) $^{+}$  RNA from Con8.hd6 cells and precipitated with 0.3 M sodium acetate and ethanol. The pellet was resuspended in 30  $\mu$ l of hybridization buffer [80% formamide, 40 mM piperazine-*N,N'*-bis(2-ethanesulfonic acid) (PIPES; pH 6.4), 400 mM NaCl, 1 mM EDTA (pH 8.0)], incubated overnight at 30°C to allow hybridization of the oligonucleotide to the RNA, and precipitated with sodium acetate and ethanol. Primer extension was performed by resuspending the pellets in 25  $\mu$ l of reverse transcriptase solution (0.5 mM each deoxynucleoside triphosphate, 50 mM Tris [pH 8.0], 5 mM MgCl<sub>2</sub>, 5 mM dithiothreitol, 50 mM KCl, 1.5  $\mu$ l of RNasin, 40 U of avian myeloblastosis virus reverse transcriptase) and incubating them for 90 min at 42°C. Reactions were extracted twice with phenol-chloroform and once with chloroform and then precipitated with ammonium acetate and ethanol. Pellets were rinsed in 70% ethanol, resuspended in Tris-EDTA, mixed with formamide loading buffer, and analyzed on a 6% sequencing gel. Unlabeled oligonucleotide (complementary to positions +45 to +79) was used to prime dideoxy sequencing from the upstream genomic construct, and this reaction was electrophoresed alongside the primer extension reaction.

**Isolation of the *sgk* genomic clone.** A Fisher rat genomic library in lambda phage (obtained from Stratagene) was probed with *sgk* cDNA at high stringency to isolate the corresponding genomic clone according to standard procedures (4). The phage DNA was digested with *HindIII*, and the fragments were electrophoretically separated on an agarose gel and transferred to nylon membranes (Schleicher & Schuell, Keene, N.H.). To identify the upstream fragment, radiolabeled +45 to +79 oligonucleotide was hybridized to the membrane-immobilized *HindIII* fragments according to standard Southern blotting procedures (30). An *sgk* genomic sequence encompassing +3513 bp to +185 bp was subcloned into pBluescript for sequence analysis. Sequencing was as described above, using overlapping restriction endonuclease subclones and complementary oligonucleotide primers. It should be noted that a 253-nucleotide intron was observed to intervene between nucleotides +140 and +141 of the *sgk* cDNA sequence.

**Construction of CAT reporter constructs.** The 4-kb genomic fragment encompassing the promoter of *sgk* was cloned into the promoterless chloramphenicol acetyltransferase (CAT) reporter gene construct pBLCAT3 (21). The 3' end was modified to terminate between the transcriptional and translational start sites, using unidirectional exonuclease III-mung bean nuclease digestions (28). Sequence analysis confirmed that the construct terminated at nucleotide +51. The construct was digested with restriction endonuclease *PstI* and religated such that nucleotides –3513 to –1610, approximately, were removed. This construct is designated –1.6 kb *sgk* pBLCAT3.

**Transfection and CAT assays.** Con8.hd6 cells in logarithmic phase were harvested with trypsin-EDTA, washed twice with phosphate-buffered saline (PBS), and resuspended in sucrose buffer (270 mM sucrose, 7 mM sodium phosphate

buffer [pH 7.4], 1 mM  $MgCl_2$ ). For electroporation, cells ( $1 \times 10^7$  to  $2 \times 10^7$  per sample in 250  $\mu$ l) were dispensed into sterile cuvettes, and plasmid DNA (25  $\mu$ g) was added. An electric pulse (400 Volts, five pulses) was delivered to the samples, using a BTX Transfectograph apparatus (BTX Inc., San Diego, Calif.). Subsequently, the cells and DNA were allowed to sit on ice for 10 min, diluted with serum-free DME-F12 (1:1) medium, and plated in 100-mm-diameter Corning plastic culture dishes. After 5 h of incubation at 37°C, the cells were refed with fresh serum-free medium either with or without dexamethasone (1  $\mu$ M). At 40 h posttransfection, the cells were harvested, washed twice in PBS, and resuspended in 0.1 M Tris-HCl (pH 7.8), and cell extracts were prepared by sonication (Branson Sonifier, model 450; VWR Scientific, San Francisco, Calif.). The cell lysates were heated at 68°C for 15 min and centrifuged at 12,000  $\times$  g, and the supernatants were recovered. The protein content of the supernatant fraction was estimated by the Bradford procedure (5). CAT activity in cell extracts containing 50 to 75  $\mu$ g of lysate protein was measured by a nonchromatographic assay (24). The enzyme assay was carried out in a final reaction volume of 250  $\mu$ l in the presence of 1  $\mu$ Ci of [ $^3H$ ]acetyl coenzyme A (specific activity, 200 Ci/mmol; DuPont/NEN); 25  $\mu$ l of 1 M Tris-HCl (pH 7.8), and chloramphenicol (50  $\mu$ l of a 5 mM aqueous solution). The reaction mixture was gently overlaid with 4 ml of a water-immiscible scintillation fluid (Econofluor; DuPont/NEN) and incubated at 37°C for 4 h. The CAT activity was monitored by direct measurement of radioactivity by liquid scintillation counting. Measurements of CAT activity were in the linear range of the assay, as determined by a standard curve using bacterial CAT enzyme. The enzyme activity was expressed as a function of protein content (counts per minute of [ $^3H$ ]acetylated chloramphenicol produced per microgram of protein per 4 h). Pure bacterial CAT enzyme (Pharmacia, Piscataway, N.J.) added to lysates of mock-transfected cells served as the positive control for the CAT enzyme assay.

**RNA analysis.** Cytoplasmic RNA was isolated from cell lines as previously described (38). To prepare total RNA from tissues, 200 mg of chopped, frozen tissue samples taken from 3-month-old Fisher 344 rats (female; except for the testes tissue) were homogenized in 2 ml of homogenization buffer (4 M guanidinium thiocyanate, 25 mM sodium citrate [pH 7.0], 0.5% sarcosyl, 0.1 M 2-mercaptoethanol) in a Polytron at low speed. One-tenth volume of 2 M sodium acetate (pH 4.0) was added, and the samples were extracted with phenol-chloroform (4:1) until the interface was clear and then once with chloroform. The RNA was precipitated once by addition of an equal volume of isopropanol and then once with 0.3 M sodium acetate and ethanol. The RNA pellet was washed in 70% ethanol, dried, resuspended in 0.1% SDS, and quantitated by measuring  $A_{260}$ . Northern (RNA) analysis of electrophoretically fractionated RNA was performed as previously described (38). Where indicated, autoradiographs were scanned with a Microtek GS400 scanner and analyzed by using the NIH Image 1.37 computer program.

**In vitro expression of *sgk*.** *sgk* cDNA cloned into pBlue-script (SK-) was linearized at the 3' end by digestion with restriction endonuclease *KpnI*, and the mRNA was transcribed in vitro with T3 RNA polymerase, using a kit from Stratagene. The RNA was capped and translated in a cell-free rabbit reticulocyte lysate system in the presence of [ $^{35}S$ ]methionine as instructed by the manufacturer (Stratagene).  $^{35}S$ -labeled molecular weight standards (Amersham)

were electrophoresed alongside in vitro translation products in SDS-10% polyacrylamide gels. Gels were fixed in 10% acetic acid and prepared for fluorography by soaking in 1 M salicylic acid for 1 h prior to drying. The dried gels were exposed to Kodak X-Omat AR film (Eastman Kodak) at -80°C.

**Production of antisera.** The peptide LGFSYAPMDSFL, corresponding to residues 419 to 431 of Sgk, with an additional N-terminal cysteine residue, was synthesized at the Microchemical Synthesis Facility, University of California, Berkeley. The peptide was coupled to rabbit serum albumin by using succinimidyl-4-[*p*-maleimidophenyl]butyrate. The rabbit serum albumin-peptide conjugate (500  $\mu$ g) was emulsified in complete Freund's adjuvant and injected subcutaneously into multiple sites of female New Zealand White rabbits. Subsequent boosts were at 4-week intervals with antigen in incomplete Freund's adjuvant. Rabbit serum was collected 10 days after each boost. The serum was repeatedly passed over a divinyl sulfone-agarose column (Sigma) conjugated with 2 mg of peptide, and after the column was washed with 50 mM Tris (pH 7.4), the affinity-purified antiserum was eluted with a buffer containing 50 mM Tris (pH 7.4), 4.5 M  $MgCl_2$ , and 1 mg of bovine serum albumin (BSA)-per ml. Antibodies were dialyzed against PBS-33% glycerol and stored at -20°C.

**Immunoblot analysis.** Con8.hd6 cells were cultured in 0.5% serum-containing medium for 36 h, and then 1  $\mu$ M dexamethasone was added for the indicated times. Cells were rinsed in PBS, harvested, resuspended in lysis buffer containing 62.5 mM Tris (pH 6.8), 1% SDS, and 10% glycerol, and then boiled for 2 min. Protein content was determined by using the Bio-Rad DC Protein Assay kit as instructed by the manufacturer. Equal amounts of protein (60  $\mu$ g) were electrophoretically fractionated through an SDS-7.5% polyacrylamide gel and transferred to nitrocellulose (NitroMEMS; MSI, Westboro, Mass.) according to standard procedures (14). Membranes were blocked overnight with 3% BSA-5% nonfat milk-0.06% Tween 20 in PBS. Primary (affinity-purified anti-Sgk) and secondary (horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G; Bio-Rad) antibody incubations were for 1 h at room temperature in 3% BSA-0.06% Tween in PBS. Washes were in 0.06% Tween in PBS, four times for 10 min each time. For immunodetection, an enhanced chemiluminescence kit (Amersham) was used, and the blots were exposed to Fuji medical X-ray film.

**Nucleotide sequence accession number.** The *sgk* cDNA sequence has been submitted to the GenBank data base and is listed under accession number U01624.

## RESULTS

**Isolation of glucocorticoid-induced genes from a Con8.hd6 cDNA library.** To isolate genes preferentially expressed in glucocorticoid-treated Con8.hd6 rat mammary epithelial tumor cells, a cDNA library was generated in lambda ZAPII phage from poly(A)<sup>+</sup> RNA isolated from cells which had been exposed to the synthetic glucocorticoid dexamethasone for 48 h. Duplicate filter lifts of approximately 50,000 plaques were hybridized with radiolabeled cDNA probes generated from mRNA isolated from either untreated (-Dex probe) or glucocorticoid-inhibited (+Dex probe) Con8.hd6 cells. Phage preferentially hybridizing to the +Dex probe were diluted and rescreened twice more until individual plaques could be isolated. One clone contained a 2.1-kb insert which hybridized to a 2.4-kb glucocorticoid-inducible transcript

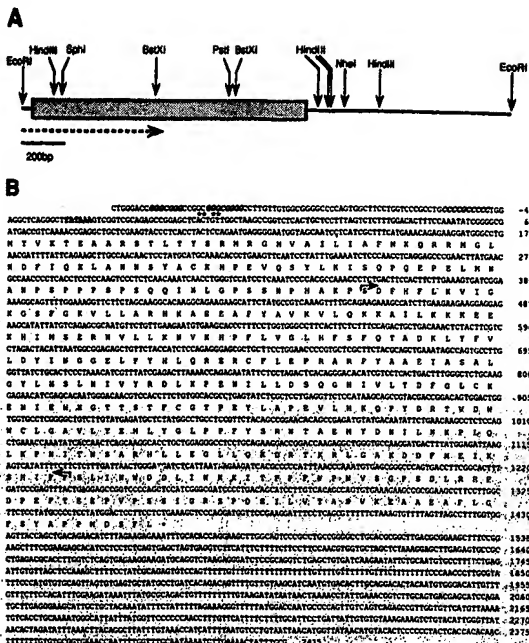


FIG. 1. Structure of *sgk* cDNA, predicted amino acid sequence, and partial promoter sequence. (A) The overall structure of the 2.4-kb *sgk* cDNA and the positions of selected restriction sites used to generate subclones for sequencing. The shaded rectangle indicates the 1.3-kb open reading frame, and the dashed arrow indicates the direction of transcription. The size bar represents 200 bp. (B) The full-length sequence of *sgk* cDNA and partial upstream promoter sequence, obtained as described in the text. The TATA box sequence is in boldface, and the three putative SP-1 binding sites in the 5' noncoding sequence are indicated by boldface italics. Asterisks indicate the four predicted transcriptional initiation sites identified from primer extension analysis. In the 3' noncoding sequence, the polyadenylation signal, 19 residues upstream of the poly(A) tail, is underlined. The arrows indicate the boundaries of the predicted serine/threonine kinase catalytic domain.

expressed in Com8.h66 cells. Comparison of partial sequence data with data in the GenBank data base showed a striking homology with several serine/threonine protein kinases. As protein kinases are fundamentally important transducers of signals for a variety of cellular processes, and glucocorticoid regulation of the message levels of a protein kinase has not been previously described, this clone was chosen for further analysis. It should be noted that RNA hybridization analysis showed that this gene was transcriptionally induced both by serum and by glucocorticoids (discussed below) and is henceforth referred to as *sgk*, for serum- and glucocorticoid-regulated kinase.

**Sequence analysis of full-length *sgk*.** The insert from the partial *sgk* clone was used to rescreen the cDNA library at high stringency; of the 12 homologous clones obtained, the longest had an insert length of 2.4 kb, which corresponded to the size of the *sgk* transcript. A partial restriction map of the full-length *sgk* cDNA is shown in Fig. 1A. The longest open reading frame (1.3 kb) predicts a 431-amino-acid protein of 49,027 Da with an isoelectric point of 9.0 (Fig. 1B). There is

no evidence for either secretory signals or nuclear localization signals in the encoded protein. There are five in-frame methionine residues encoded within the first 150 nucleotides; however, the methionine designated as the first amino acid in Fig. 1B is within an appropriate context for translation initiation (19). To determine the size of the polypeptide encoded by *sgk*, *sgk*-specific transcripts were synthesized in vitro from the full-length cDNA and translated in a cell-free system in the presence of [<sup>35</sup>S]methionine. Consistent with the predicted open reading frame, the predominant radiolabeled protein has an apparent molecular size of 49 kDa (Fig. 2, lane B).

To identify the start site of the *sgk* transcript and to begin to characterize its promoter elements, the *sgk* cDNA was used to screen a rat genomic phage library to isolate the corresponding genomic clone. By primer extension analysis, transcription was shown to initiate at four distinct nucleotides, as indicated in Fig. 3. Partial sequence analysis of a restriction fragment, obtained from a rat genomic library, that contains the *sgk* promoter revealed a TATA box ele-

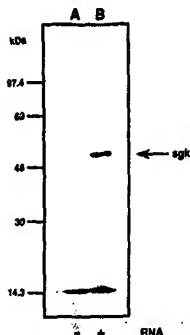


FIG. 2. In vitro transcription-translation analysis of *sgk*. cDNA containing full-length *sgk* was linearized at the 3' end and transcribed in an in vitro system. Either 1  $\mu$ g of the in vitro-sequenced RNA (lane B) or no RNA (lane A) was added to a rabbit reticulocyte lysate system and translated in vitro in the presence of [ $^{35}$ S]methionine as described in the text. Translation products were reduced and denatured and then electrophoresed through an SDS-10% polyacrylamide gel, and radiolabeled protein bands were visualized by autoradiography. The mobilities of [ $^{35}$ S]-labeled molecular weight standards are indicated. The arrow indicates the predominant band at the predicted molecular size of *sgk*-encoded protein (49 kDa).

ment centered 25 nucleotides upstream of the transcriptional start sites (6) and several SP-1 consensus sites at approximately -45, -100, and -110 (7) (Fig. 1A).

**Comparison of *sgk* with other serine/threonine protein kinases.** The catalytic domains of protein kinases are composed of approximately 270 amino acids, within which 11 distinct subdomains are recognized (12). Within subdomain I (Fig. 4), *sgk* possesses the characteristic ATP-binding motif Gly-X-Gly-X-X-Gly, with a conserved Lys 17 residue downstream. The highly conserved sequences Asp-Phe-Gly in subdomain VII and Ala-Pro-Glu in subdomain VIII are also indicative of a functional protein kinase. The presence in subdomain VII of the sequence Asp-Leu-Lys-Pro-Glu-Asn and in subdomain VIII of the Gly-Thr-X-X-Tyr-X-Ala-Pro-Glu motif strongly suggests specificity towards serine/threonine substrates (13). Comparison of the predicted catalytic domain of *sgk* with those of other protein kinases revealed almost equal homology with the *rac* kinases (54% identity with human *rac-a* (18)), the protein kinase C family (48% identity with rat protein kinase C- $\beta$  (16)), ribosomal protein S6 kinase (50% identity with the rat protein (20)), and cyclic AMP (cAMP)-dependent protein kinase (45% identity with the mouse catalytic subunit (36)), indicating that *sgk* is a distinct kinase which cannot be easily assigned to any related serine/threonine kinase family. The alignments of selected catalytic domains are shown in Fig. 4. The sequence upstream of the catalytic domain bears no obvious sequence homology with these kinases or with other known proteins.

**Tissue- and species-specific expression of *sgk*.** As *sgk* cDNA had been isolated from a rat mammary epithelial tumor cell line, the tissue and species distribution of *sgk* mRNA was tested in a variety of cell types. Tissues from young adult Fisher 344 rats were removed, and total RNA was isolated and examined for the expression of *sgk*. As shown in Fig.

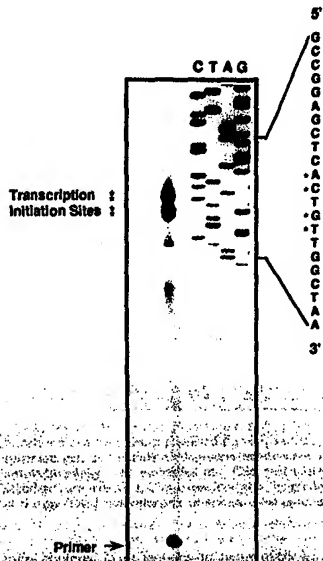


FIG. 3. Primer extension analysis to determine the start sites of transcription of *sgk*. An oligonucleotide complementary to nucleotides +45 to +79 of *sgk* was end labeled with [ $\gamma$ - $^{32}$ P]ATP, hybridized to 10  $\mu$ g of Con8.hdb6 poly(A)<sup>+</sup> RNA, extended with avian myeloblastosis virus reverse transcriptase as described in the text, and electrophoresed through a 6% denaturing polyacrylamide gel (left lane). The positions of the extended primer and the four transcription initiation sites defined by this analysis are indicated by an arrow and asterisks, respectively. To determine the initiating residues precisely, unlabeled primer was used to prime a dideoxynucleotide sequencing reaction from the *Hind*III genomic fragment encoding the promoter sequences. This reaction was electrophoresed alongside the primer extension reaction (right lanes). The lane designations and expanded sequence shown at right indicate the complementary nucleotides (i.e., sense strand) to the actual sequence read from the gel.

**5A. *sgk* expression has a distinct pattern of tissue specificity,** with highest levels found in the ovary, thymus, and lung and low but detectable levels found in most other tissues, including the mammary gland from which the Con8.hdb6 tumor cell line was derived. *sgk* expression and glucocorticoid induction were examined in several types of normal and transformed cell lines (Fig. 3B) grown in the presence or absence of dexamethasone for 48 h. In the three rat cell lines examined, Rat2 fibroblasts, HTC hepatoma tumor cells, and Con8.hdb6 mammary cells, *sgk* message was present and highly inducible, although the highest basal and induced levels were observed in the mammary tumor cells. In the cell lines of mouse (NMuMg) and human (HeLa) origin, a 2.4-kb message was also detected and was inducible by glucocorticoids, indicating the presence of an *sgk* homolog in other

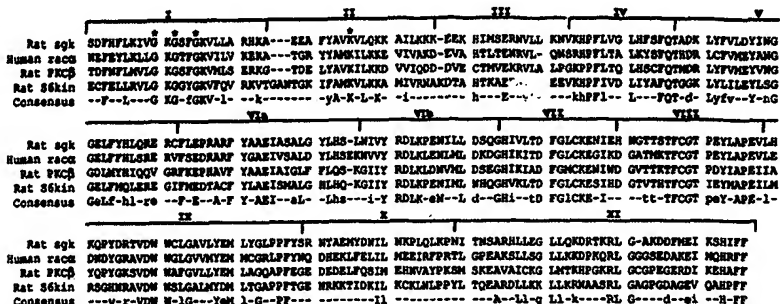


FIG. 4. Alignment of *sgk* with the catalytic domains of selected serine/threonine protein kinases. The catalytic domains and subdomains (I to XI) (13) of *sgk*, human *rac*, rat protein kinase C- $\beta$  (pPKC), and rat S6 kinase (S6 kin) (see text for references) were aligned by using the Intelligenetics Genalign program. In the consensus sequence, capital letters refer to identity across all four sequences. Lowercase letters indicate identity of *sgk* with two of the three other sequences. The consensus residues of the ATP-binding domain are designated by asterisks. The one-letter amino acid code is used.

species. There was no apparent correlation between glucocorticoid inhibition of proliferation and induction of *sgk* transcripts; for example, the proliferation of HeLa cells, HTC cells, and untransformed fibroblasts was not affected by glucocorticoids (data not shown), but each cell line

expressed high levels of dexamethasone-inducible *sgk* mRNA.

**Glucocorticoids directly stimulate *sgk* mRNA expression.** To determine whether the glucocorticoid induction of *sgk* transcripts is an immediate and direct response, the time course of the dexamethasone-mediated increase in *sgk* transcript levels was examined in Con8.h66 mammary epithelial cells. Cells were grown in low-serum medium for 36 h, and RNA was isolated at various times following addition of dexamethasone or of dexamethasone plus the protein synthesis inhibitor cycloheximide. Within 30 min of dexamethasone treatment, *sgk* expression was stimulated by twofold relative to untreated cells (Fig. 6A, top). This increase was independent of de novo protein synthesis, as shown by similar levels of *sgk* mRNA at 30 min in the presence of cycloheximide. Transcript levels continued to accumulate for several hours in dexamethasone-treated cells, reaching 23-fold higher than background by 24 h, and were slightly higher throughout in the presence of cycloheximide. The ability of dexamethasone to rapidly induce *sgk* transcripts in the absence of de novo protein synthesis was also examined in other cell types, such as Rat2 untransformed fibroblasts, with comparable results (data not shown). The increase in *sgk* mRNA in the rat mammary tumor cells was shown to be a result of new synthesis rather than simply an increase in mRNA stability, since the induction by dexamethasone at 1 h was completely blocked in the presence of 5  $\mu$ g of the transcription inhibitor actinomycin D per ml (Fig. 6B). Furthermore, *sgk* induction is a glucocorticoid receptor-dependent response, since other steroids (cholesterol, progesterone,  $\beta$ -estradiol, and testosterone) were unable to mimic the dexamethasone response, whereas hydrocortisone and corticosterone, which act through the glucocorticoid receptor, increased *sgk* transcripts to high levels (Fig. 6C).

**Serum stimulation of *sgk* expression.** During the course of our studies, we observed that *sgk* transcripts were always elevated when the mammary tumor cells were cultured in serum-supplemented medium. To characterize this response, cells were first cultured in low-serum medium, and

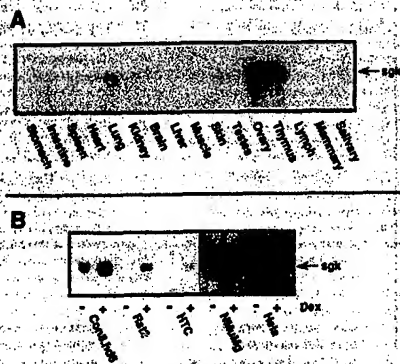


FIG. 5. Tissue and cell type specificity of *sgk* expression. (A) Total RNA was isolated from tissues obtained from young Fisher 344 rats (female, except for the testes); 10  $\mu$ g was examined by Northern blot analysis for expression of the 2.4-kb *sgk* transcript (arrow) as described in the text. (B) Cell lines were cultured for 48 h in the absence (– Dex) or presence (+ Dex) of 1  $\mu$ M dexamethasone in medium containing 10% calf serum, harvested at subconfluence, and examined by Northern blot analysis for *sgk* transcripts. The autoradiographs were differentially exposed: Con8.h66 (rat transformed mammary), Rat2 (rat fibroblast), and HTC (rat hepatoma) lanes were exposed to X-ray film overnight, whereas NMuMG (mouse untransformed mammary) and HeLa (human cervical carcinoma) lanes were exposed for 10 days.



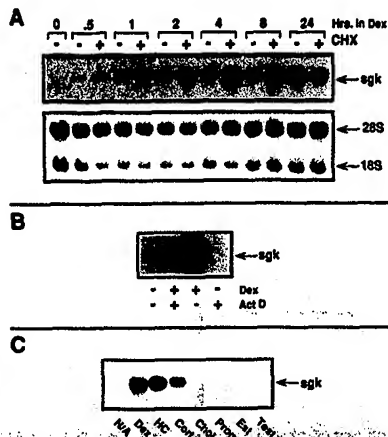


FIG. 6. Dexamethasone induction of *sgk* transcripts in Con8.hd6 mammary tumor cells. (A) In the upper panel, Con8.hd6 cells maintained in low serum medium for 36 h were treated with dexamethasone (1  $\mu$ M) at time 0 for the indicated periods (Hrs. in Dex) with (+ CHX) or without (- CHX) the addition of 10  $\mu$ g of the protein synthesis inhibitor cycloheximide per ml; 10  $\mu$ g of cytoplasmic RNA from each time point was examined for *sgk* expression by Northern blot analysis as described in the text. To demonstrate equivalent loading of undegraded samples (lower panel), the filter used for *sgk* detection was stained with methylene blue following RNA transfer. The 28S and 18S ribosomal bands are indicated. (B) Con8.hd6 cells were treated for 1 h with the indicated combinations of dexamethasone (Dex; 1  $\mu$ M) and actinomycin D (Act D; 5  $\mu$ g/ml). Cytoplasmic RNA was isolated, and Northern blots probed for *sgk* transcripts as described above. (C) Con8.hd6 cells were treated for 24 h with the indicated steroids at a concentration of 1  $\mu$ M; RNA was isolated, and *sgk* transcripts were examined by Northern blot analysis as described above. Abbreviations: N/A, no addition; Dex, dexamethasone; HC, hydrocortisone; Cori, corticosterone; Chol, cholesterol; Prog, progesterone; Est,  $\beta$ -estradiol; Test, testosterone.

RNA was isolated at various times following exposure to 10% serum. As shown in Fig. 7, *sgk* transcript levels were strongly stimulated within 30 min of exposure to serum, suggesting that induction of *sgk* is an immediate response to serum in the Con8.hd6 mammary tumor cells. In the continuous presence of 10% serum, *sgk* transcript levels continued to accumulate for 4 h and then remained at the same high level at later time points. Densitometry of the Northern blot shown in Fig. 7A revealed that *sgk* transcripts are maximally induced approximately eightfold by serum. The effect of serum was not due to any residual glucocorticoids, since the same induction profile was observed in the presence of the powerful glucocorticoid antagonist RU38486 (data not shown).

The relative effects of dexamethasone and serum on the induction of *sgk* transcripts were compared in the presence or absence of de novo protein synthesis. Con8.hd6 mammary tumor cells grown in low serum were treated with or without cycloheximide and then incubated with the indicated

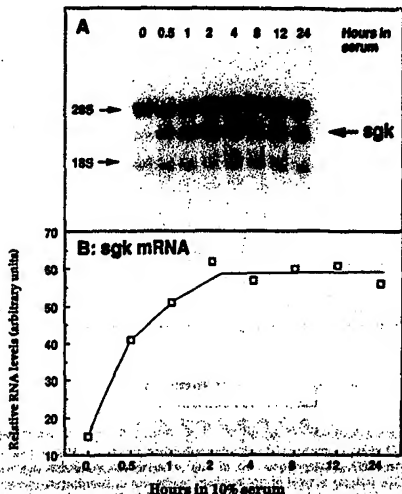


FIG. 7. Time course of induction of *sgk* transcripts by serum in Con8.hd6 mammary tumor cells. (A) Con8.hd6 cells maintained in low serum for 36 h were treated with 10% serum; at the indicated times, total cytoplasmic RNA was isolated, and 10  $\mu$ g of RNA was examined for *sgk* expression by Northern blot analysis. Equivalent loading at each time point is shown by the 28S and 18S ribosomal bands which, nonspecifically hybridized with the radiolabeled *sgk* cDNA probe. (B) The fluorogram displayed in panel A was scanned with a Microtek GS400 scanner, using the NIH Image 1.37 program to determine the relative levels of *sgk* transcripts produced at each time point.

combinations of 1  $\mu$ M dexamethasone and 10% serum for 2 h. Isolated RNA was electrophoretically fractionated, and Northern blots were hybridized with an *sgk*-specific cDNA probe. As shown in Fig. 8, dexamethasone induced *sgk*

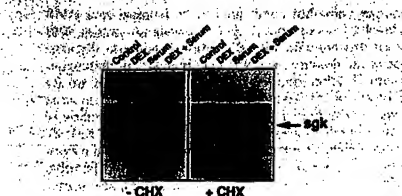


FIG. 8. Effects of cycloheximide on the induction of *sgk* transcripts by dexamethasone and/or serum. Con8.hd6 cells maintained in low serum for 36 h were treated with the indicated combinations of 1  $\mu$ M dexamethasone (DEX) and 10% serum with (+ CHX) or without (- CHX) the addition of 10  $\mu$ g of the protein synthesis inhibitor cycloheximide per ml. After 2 h of incubation, total RNA was isolated and examined for *sgk* mRNA expression by Northern blot analysis.



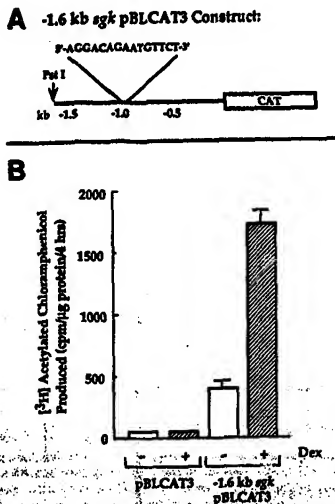


FIG. 9. Glucocorticoid responsiveness of the *sgk* promoter. (A) The promoter of *sgk*, encompassing -1.6 kb to +51 bp relative to the start of transcription, was fused upstream of the CAT reporter gene to generate the -1.6 kb *sgk* pBLCAT3 construct. The relative position of the GRE consensus element in the promoter is indicated. (B) The promoterless construct pBLCAT3 or construct -1.6 kb *sgk* pBLCAT3 was transfected by electroporation into Con8.hdb cells. Following incubation with or without dexamethasone (Dex), cells were harvested and CAT activity was examined as described in the text.

transcripts to a slightly greater extent than did serum. Treatment with both reagents was additive, suggesting that glucocorticoids and serum induce *sgk* mRNA by independent mechanisms. Furthermore, exposure to cycloheximide did not affect the magnitude of stimulation of *sgk* transcripts by either glucocorticoids or serum (Fig. 8), which is consistent with the regulation of *sgk* being a direct response to each reagent.

*sgk* possesses a functional GRE in its promoter. The results presented above indicated that *sgk* transcription is directly and specifically regulated by glucocorticoids, suggesting the existence of a GRE in its promoter. Analysis of the genomic sequence upstream of the transcriptional start of *sgk* revealed a sequence (5'-AGGACACAATGTTCT-3') with close homology to the consensus GRE (5'-GGTACAnnTGT-TCT-3') (2) at approximately -1 kb. To confirm that this GRE was functional, the *sgk* promoter sequence from approximately -1.6 kb to +51 bp was ligated upstream from the bacterial CAT reporter gene (Fig. 9A). This reporter plasmid or a control promoterless construct was transfected into Con8.hdb cells, and the relative CAT activity was determined following incubation with or without dexamethasone for 40 h. As seen in Fig. 9B, in the absence of dexamethasone, the -1.6 kb *sgk* pBLCAT3 construct stim-

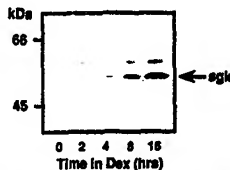


FIG. 10. Expression of *sgk* protein in response to glucocorticoids. Con8.hdb cells were grown in 0.5% serum-containing medium for 36 h, and then dexamethasone (Dex; 1  $\mu$ M) was added for the indicated times. Cells were harvested and lysed, and equal amounts of cellular proteins were electrophoretically fractionated through an SDS-7.5% polyacrylamide gel and transferred to nitrocellulose filters. Filters were blocked overnight and then incubated with affinity-purified *sgk* antibodies and horseradish peroxidase-conjugated secondary antibodies, and immunoreactive proteins were detected by an enhanced chemiluminescence detection system.

ulated CAT activity sevenfold over the promoterless pBLCAT3 construct, indicating that the endogenous promoter is active under serum-free conditions. In the presence of dexamethasone, there was a further fivefold stimulation of CAT activity, consistent with the GRE-like element in the *sgk* promoter being functional and suggesting that enhancement through this element could account for the observed transcriptional regulation of *sgk* by glucocorticoids. To date, we have been unable to detect a functional serum response element with use of *sgk*-CAT reporter plasmids containing various lengths of *sgk* 5' flanking sequences.

*sgk* protein levels correspond to RNA levels. To determine whether the dexamethasone induction of *sgk* mRNA results in a concomitant stimulation of *sgk*-encoded protein, antibodies were generated against a synthetic peptide corresponding to its predicted carboxy-terminal 14 amino acids. At various times following treatment of Con8.hdb cells with dexamethasone, cells were lysed and equal amounts of cellular proteins were electrophoretically fractionated through an SDS-polyacrylamide gel. The proteins were transferred to nitrocellulose and probed first with affinity-purified *sgk* antisera and then with horseradish peroxidase-conjugated secondary antibodies; and the immunoreactive proteins were visualized by using an enhanced chemiluminescence detection system. As shown in Fig. 10, and consistent with the glucocorticoid-mediated induction pattern of *sgk* transcripts, the stimulation of a 49-kDa band was observable as early as 2 h following dexamethasone addition. The level of this protein continued to increase in the presence of dexamethasone and was 10-fold higher than the uninduced level by 16 h. To confirm that this 49-kDa protein is indeed *sgk*, the peptide used to generate the antiserum was able to abolish the band, whereas a nonspecific peptide had no effect (data not shown). Furthermore, preimmune antiserum did not reveal an immunoreactive protein band at 49 kDa from either untreated or dexamethasone-treated cells (data not shown). This finding indicates that the induction of *sgk* protein closely corresponds with the transcriptional regulation of *sgk* by dexamethasone.

## DISCUSSION

We have cloned and characterized the cDNA for a novel member of the serine/threonine protein kinase gene family, designated *sgk*, which has approximately 50% identity with

the catalytic domains of several important signal-transducing protein kinases, including protein kinase C and cAMP-dependent kinase. Induction of the 2.4-kb transcript for *sgk* requires glucocorticoid receptor-binding steroids, occurs within 30 min of dexamethasone treatment, and is independent of de novo protein synthesis. Consistent with the stimulation of *sgk* being a primary response to glucocorticoids, sequencing of genomic DNA revealed an apparent GRE in the promoter of *sgk*. Indeed, the expression of a transfected CAT reporter gene was stimulated by dexamethasone when fused to a segment of the 5' flanking sequence of *sgk* which includes the GRE. This study is the first report of transcriptional regulation by glucocorticoids of a gene encoding a presumed protein kinase.

*sgk* transcripts are also rapidly and strongly induced by serum. Similar to the glucocorticoid induction profile, *sgk* mRNA is elevated within 30 min of serum treatment in a process that does not require de novo protein synthesis. Only one other serum-inducible kinase gene, *snk*, has been reported (32), although the effects of serum are blocked by glucocorticoids. In contrast to *snk*, glucocorticoids and serum additively stimulate *sgk* transcript levels, suggesting that these two regulators act through independent signal transduction pathways. It is interesting to consider that *sgk* and *snk* may represent the first two identified protein kinases of a group of kinases which are under rapid transcriptional control; as opposed to posttranslational control as observed for the majority of kinases. We are currently examining *sgk* 5' flanking sequences for regulatory elements which can account for the serum stimulation of its transcript levels as well as testing the effects of added polypeptide growth factors.

We have not yet been successful in demonstrating enzymatic activity for *sgk*, although several different approaches have been attempted. In vitro translation of *sgk* mRNA showed no obvious *sgk*-dependent total or immunoprecipitable protein kinase activity, using histone H1 and myelin basic proteins as substrates in a standard protein kinase assay. We were also unable to detect protein kinase activity in in vitro-translated *sgk* epitope tagged with polyomavirus middle T-antigen or in bacterial fusion proteins produced with *sgk* linked to glutathione S-transferase. It is possible that the lack of detectable protein kinase activity reflects *sgk*'s requirement of a second subunit or other critical cofactors missing in the kinase assay. Furthermore, depending on its function, *sgk* may have a restricted set of substrates. It is also possible that *sgk* does not have any enzymatic activity; however, the predicted amino acid sequence for *sgk* strongly suggests that it is a functional serine/threonine protein kinase. We propose that *sgk* through phosphorylation of specific target proteins, may be one of the secondary mediators of a subset of cellular responses to glucocorticoids and serum. It will therefore be important to determine *sgk*'s precise function and physiological substrates.

Protein phosphorylation is a rapid and reversible means of transducing signals from the extracellular environment that lead to pleiotropic cellular responses (27, 39). Protein kinases characterized to date are primarily regulated at a posttranslational level, both by phosphorylation and by interaction with small regulatory molecules. Several serine/threonine kinases related to *sgk* possess pseudosubstrate sequences either within the same polypeptide chain (as for protein kinase C) or in a separate subunit (as for cAMP-dependent protein kinase) which normally block the substrate- and ATP-binding sites of the catalytic domain and

maintain the kinase in an inactive form (23, 33, 34). The autoinhibitory domain is released in response to second messengers generated by extracellular signals, which results in the rapid activation of kinase activity. The short noncatalytic amino-terminal domain of *sgk* has no apparent homology with domains of known genes, including the regulatory domains of other protein kinases. We speculate that modulating the level of this protein by hormonally regulated transcription factors may be the primary means of regulating its enzymatic activity.

Glucocorticoid receptors are present in virtually all mammalian cell types, and there is a diversity of physiological processes affected by hormonal treatment, including metabolism, differentiation, inflammation, and proliferation (3, 22). There appears to be no clear correlation between growth inhibition of cell lines in vitro by glucocorticoids and induction of *sgk* transcripts, suggesting that *sgk* may not be sufficient to mediate the hormone-dependent suppression of proliferation that was observed in Con8.hd6 cells (37, 38). We are currently attempting to understand the precise relationship between *sgk* activity and the control of cellular proliferation. *sgk* mRNA was shown to be present at highest levels in normal thymus, ovary, and lung tissue, predicting a physiological role for *sgk*-encoded kinase activity in these tissues. While the significance of this tissue-specific expression pattern is unclear, it is interesting to note that the proto-oncogene *c-akt*, which has strong sequence similarity to *sgk*, was also reported to be most abundantly expressed in the normal thymus (4).

Transcriptional induction of a related kinase, protein kinase C- $\beta$ , by a secosteroid hormone, 1,25-dihydroxyvitamin D<sub>3</sub>, has been reported (25), although this induction was neither as immediate nor as dramatic as the effect of glucocorticoids on *sgk*. It is tempting to speculate that steroid regulation of protein kinase transcription could be a general mechanism for regulating specific phosphorylation networks. The direct transcriptional induction of a protein kinase by glucocorticoids and serum, as demonstrated here for *sgk*, may reflect a previously undescribed cross-talk between a membrane-linked signaling pathway and intracellular steroid hormone activation of gene transcription. We are currently extending our work to determine the immediate substrates for *sgk* and the role of its enzymatic activity in glucocorticoid- and serum-mediated cellular events.

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